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Fertilization with Sexed Equine Spermatozoa Using Intracytoplasmic Sperm Injection and Oviductal Insemination

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Currently, the success of in vitro embryo production in the horse lags behind other domestic species. Barriers to routine in vitro fertilization (IVF) include oocyte maturation and sperm capacitation. Follicular fluid supplementation of medium as well as addition of progesterone to medium has been shown to improve maturation equine oocytes. Intracytoplasmic sperm injection (ICSI) has been used in humans as an alternative to conventional in vitro fertilization. Del'Aquila et al. (1996, 1997) reported a higher fertilization rate using ICSI than conventional IVF.

Offspring of several species have been produced using semen that has been sorted for X- and Y-chromosome-bearing spermatozoa. However, only low numbers of viable spermatozoa sorted for X- and Y-bearing chromosomes can be attained with flow cytometry. Thus, techniques such as in vitro fertilization, ICSI and oviductal insemination are necessary in order to use X- and Y-sorted sperm. The objective of this study was to evaluate the effect of follicular fluid and progesterone on oocyte maturation as assessed by embryo development after sperm injection, and to determine if equine pregnancies could be produced with X- and Y-sorted sperm using intracytoplasmic sperm injection and oviductal insemination.

Compact cumulus oocyte complexes (COC) were cultured in 1 of 3 treatment groups: 1) Hepes-buffered TCM-199 with 10% estrous cow serum (ECS) plus estradiol, LH and FSH (control medium); 2) control medium with 20% follicular fluid; and 3) control medium with 250 ng/ml progesterone. Oocytes were cultured for 36 to 40 hr and those with first polar bodies were fertilized by ICSI. There was no difference among treatments in the percent of oocytes with first polar body. However, maturation of oocytes was greater for in-vivo matured than in-vitro matured oocytes. Oocytes with expanded cumulus-oocyte complexes were matured 36-43 hr in Hepes-buffered TCM-199 with 10% estrous cow serum (ECS), estradiol, LH and FSH with either 50, 250 or 1,250 ng/ml of progesterone. Oocytes with a visible first polar body were fertilized using ICSI and cultured at 39°C in 5% CO_2/5% O_2/90% N_2 in a chemically-defined medium + 1% BSA and 0.5 mM fructose for 72 hr, then with 5.0 mM fructose for an additional 48 hours. Embryos resulting from ICSI were transferred nonsurgically at the morula stage.

High levels of progesterone (1,250 ng/ml) resulted in lower oocyte maturation. Overall, 50.5% of the oocytes developed a first polar body. Of those injected with sperm, there was no difference in embryonic development among treatments. Thirty-two COCs were matured in Hepes-buffered TCM-199 with estradiol, LH and FSH containing 0, 50 or 250 ng/ml of progesterone. After 40-43 hr in maturation medium, oocytes with first polar bodies were injected with X-chromosome-bearing spermatozoa. Sex-sorted sperm were incubated in 38 µM Hoechst 33342 at a concentration of 75 x 10^6 spermatozoa/ml in TALP for 1 hr at 34°C and then sorted based on DNA content using a Cytomation MoFlo® flow cytometer/cell sorter modified for sexing sperm. Sorted sperm were centrifuged at 400 x g for 10 min through a 1.5-ml column of 20% BSA

and TALP and then resuspended in TALP for ICSI. For ICSI, oocytes were held in modified human tubal fluid plus 1% fetal calf serum and sperm were held in modified human tubal fluid plus 10% BVP under oil. Once injected, oocytes were moved into a droplet of culture medium with 10 μM calcium ionophore for 5 min for activation. Oocytes were cultured in a chemically-defined medium after sperm injection.

Thirteen injected oocytes developed to the 2- to 3-cell stage, 8 to the 4- to 6-cell stage and 2 oocytes developed to the 7- to 8-cell stage. One embryo was transferred at the 7- to 8-cell stage, but no pregnancy resulted. Seven of the embryos produced from sperm injection of X-bearing spermatozoa were analyzed by PCR and verified to be female. Oviductal insemination was performed 24 to 48 hr after hCG injection by standing flank laparotomy. A 50-μl sample of sperm containing approximately 150,000 sex-sorted X-bearing sperm were deposited. Oviductal insemination by cannulation of the fimbriated end of the oviduct (n=2) resulted in 1 ongoing pregnancy.

These studies apparently report the first embryos and pregnancy from equine semen sexed by flow cytometry.

NOTES:

DETAILED ACTION

1. This action is in response to the amendment filed June 18, 2003. Applicants arguments and amendments have been fully considered but are not persuasive to overcome all grounds of rejection. All rejections not reiterated herein are hereby withdrawn. This action is made final.

Information Disclosure Statement

2. In the Information Disclosure Statement filed June 18, 2003, the non-English reference of Linge et al has not been considered fails to include a concise statement of the relevance of the following non-English language reference listed, as required under 37 CFR § 1.98(a)(3). In the response of June 18, 2003, Applicants state that a concise explanation of this reference was included with the response. However, the response did not include statement of the relevance of the Linge reference. Applicants submitted only the originally filed non-English version of the Linge reference.

Claim Rejections - 35 USC § 103

3. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein

were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 45-47 and 49-51 are rejected under 35 U.S.C. 103(a) as being unpatentable over Rens (U.S. Patent No. 5,985,216) in view of Wilhelm (Cryobiology (1996) 33:320-329). This rejection now applies to newly added claims 136 and 137.

Rens teaches a method of high speed flow cytometry for sorting sperm. In the method of Rens (see columns 4-6), a sample of sperm is obtained from a male mammal, the sperm is stained with Hoeschst 33342 dye in order to distinguish between viable and nonviable sperm (column 5, lines 4-10), the sperm are sorted in a high speed flow cytometer using a nozzle that forms a stable droplet containing each individual sperm cell (column 2, lines 23-32), the sperm are sorted according to their sex characteristics and isolated populations of X- and Y-chromosome bearing sperm are collected. Approximately 50% of the sperm were viable and the sorting was performed at sampling rates of 500 sperm/sec and 2000 sperm/sec (see column 6). Further, the nozzle allowed for sample rates up to at least 15,000 sperm/sec (column 4, lines 29-31). Rens exemplifies using the claimed sorting method using rabbit, bull, mouse and human sperm (columns 4-7) and states that the sorting method can be used with any mammalian sperm (column 4, lines 38-42). Rens does not specifically exemplify applying the sorting method to equine sperm.

However, Wilhelm teaches the use of equine sperm for the purpose of artificial insemination. It would have been obvious to one of ordinary skill in the art at the time the invention was made to have applied the method of Rens to the sorting of equine sperm in order to have provided an effective means for distinguishing between and collecting populations of X- and Y-chromosome bearing sperm useful for artificially inseminating equine.

Secondly, Rens does not specify the solution into which the sperm cells are collected. However, Wilhelm teaches extending equine sperm in skim milk solution containing 2% egg yolk by volume (page 322; referred to therein as SMEY). Wilhelm teaches that SMEY extender effectively preserves equine sperm during freezing and thawing. Accordingly, it would have been obvious to one of ordinary skill in the art at the time the invention was made to have further modified the method of Rens so as to have collected the equine sperm in the SMEY solution of skim milk and egg yolk in order to have provided an medium that could be used for freezing and then thawing the sorted equine sperm, thereby providing sorted equine sperm that could be used more effectively for artificially inseminating female equine. With respect to claim 47, the specification has not established any unexpected results with using 4% egg yolk and the recitation of "about four percent egg yolk" is considered to encompass 2% egg yolk. Furthermore, it would have been well within the skill of the art at the time the invention was made to have modified the concentration of egg yolk in the extender solution in order to have provided the most effective concentration of egg yolk depending on the other reagents present in the extender solution.

With respect to claim 51, Rens does not specify the pressure used to operate the high speed cell sorter. However, methods for sorting equine sperm using high speed cell sorters were well known in the art at the time the invention was made. To determine the optimum conditions for performing a method step is well within the skill of the art. As discussed in MPEP 2144.05(b), "(w)here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation. *In re Aller,* 220 F.2d 454, 105 USPQ 233, 235 (CCPA 1955). Further, with respect to claims 136 and 37, Rens teaches that 4 to 5 million sorted sperm were used to inseminate dairy cows, but does not teach the quantity and volume of sperm in equine artificial insemination sample. However, since the parameters which effect artificial insemination of equine were known in the art at the time the invention was made, it would have been obvious to one of ordinary skill in the art and well within the skill of the art to have selected an optimum quantity of sperm, wherein said quantity would be less than 25 million and to have selected the optimum volume for the artificial insemination sample, so as to have provided the most effective sample for inseminating equine while keeping the number of sperm to be used for insemination at the lowest possible number given the constraints on how many sperm could be sorted per day and the cost of sorting.

4. Claims 48 is rejected under 35 U.S.C. 103(a) as being unpatentable over Rens in view of Wilhelm and further in view of Catt (cited in the IDS of January 29, 2001).

The teachings of Rens and Wilhelm are presented above. The combined references do not teach establishing a sheath fluid which contains a HEPES buffered

medium. Catt teaches that semen may be diluted in a HEPES-buffered SOF (synthetic oviduct fluid) medium and that such a fluid is suitable for maintaining the viability of spermatozoa (see, e.g., page 252 and 257). Catt also teaches that it is beneficial to sort into a medium containing a cushioning of seminal plasma to increase the viability and motility of sperm. Accordingly, it would have been obvious to one of ordinary skill in the art at the time the invention was made to have further modified the method of Rens in view of Wilhelm so as to have used a HEPES-buffered medium for establishing a sheath fluid because Catt teaches that this is a suitable dilution medium for sperm and thereby using HEPES-buffered medium as the sheath fluid would have achieved the benefit of ensuring the viability and motility of the sperm.

5. **RESPONSE TO ARGUMENTS:**

In the response filed June 18, 2003, Applicants did not traverse the rejections separately but rather argued both of the 103 rejections together. Applicants traverse the rejections by arguing that there is a distinction between "living sperm cells" and "viable sperm cells". Applicants assert that Rens only compares motile sperm cells, non-motile sperm heads and dead sperm cells. These arguments have been fully considered but are not persuasive because Applicants are arguing limitations not recited in the claims. The claims do not require the sorting of or the establishment of an insemination sample containing any particular number of live or viable sperm. In fact, the claims do not even mention live or viable sperm. Accordingly, these arguments do not relate to the present claims.

Applicants further traverse the rejection by stating that Rens does not teach that the sperm cells are sorted at rates of 500 sperm per second or at 2000 sperm per second. However, the Office action did not indicate that Rens taught such a limitation. As stated in the Office action, Rens teaches sampling rates of 500 sperm/second and 2000 sperm/second. It is argued that the sample rate as taught by Rens is entirely different from obtaining approximately 1000 live sperm cells of each sex chromosomal composition as described and claimed by Applicants. However, Applicants do not in fact claim methods of obtaining 1000 live sperm cells of each sex chromosomal composition. Rather, claim 51 is dawn to a method using a sorter in which the pressure is at least about fifty pounds per square inch. Claim 50 is limited to methods comprising "sorting said droplets having said equine sperm cells entrained at a rate of a least nine hundred per second". The claim does not require obtaining 1000 live sperm cells. Regardless, Applicants have not shown that the method of Rens would not result in the sorting of sperm at a rate of at least 900 sperm per second.

Applicants discuss the distinction between "sexed spermatozoa" and "naturally occurring sperm cells." It is argued that non-naturally occurring sperm cells have a very low vigor when conventionally sorted. It is stated that the spermatozoa taught by Wilhelm are naturally occurring spermatozoa and are treated in a completely different manner than the method claimed. However, Applicants arguments do not pertain to the present grounds of rejection. Wilhelm was not cited for teaching the sorting of sperm according to the method of the claimed invention. Wilhelm was cited as teaching the advantages of artificially inseminating equine. Rens, on the other hand, was cited for its

teachings of methods for sorting sperm based on their sex characteristics in order to obtain sperm samples useful for artificial insemination and the application of this methodology to any mammalian sperm sample.

Applicants state that "the Catt reference does not teach the use of HEPES buffered medium as a sheath fluid." However, Catt does in fact teach that semen may be diluted in a HEPES-buffered SOF (synthetic oviduct fluid) medium and that such a fluid is suitable for maintaining the viability of spermatozoa (see, e.g., page 252 and 257).). Catt further teaches that it is beneficial to sort into a medium containing a cushioning of seminal plasma to increase the viability and motility of sperm.

It is argued that the combination of references do not provide the requisite suggestion or motivation for combining references. The examiner disagrees. Rens teaches that the method of sorting sperm is applicable to any sample of mammalian sperm. This is a very clear statement of motivation to apply the method to any other mammalian sperm sample and one of skill in the art would immediately recognize that equine are encompassed by the stated group of mammals.

Applicants state that the combined references do not teach using a flow cytometer at a pressure of 50 psi. It is stated that the higher pressure allows sort rates of 900 sperm per second. However, as set forth in the office action, Rens is silent with respect to the operating pressure. Rens also specifies the sampling rate but does not specify the sorting rate. However, the rejection was based on the fact that it was within the skill of the ordinary artisan art at the time the invention was made and it would have been obvious to one of ordinary skill in the art at the time the invention was made to

have selected the optimum pressure for operating the flow cytometer. The conclusions drawn by Applicants in this response do not overcome the rejection. There is no evidence to show that one of ordinary skill in the art would not have in fact selected similar pressures for operating the sorter, since as stated by Applicants using lower pressures would have resulted in a sorting process that would take at least 34 hours and in this time the majority of the sperm cells would have been dead. Wouldn't one of ordinary skill in the art have recognized this? If Applicant's methods do in fact require operating the flow cytometer at pressures well above those conventionally used in the art and what those in the art would have recognized as the pressure one would use in the method of Rens, then Applicants should have provided evidence to support such arguments in their present response.

Applicants state that "Importantly, the combination of references does not disclose even a single pregnancy of any mammal from insemination with sorted spermatozoa." Firstly, it is noted that the rejection was made under 35 USC 103 and not 102 and there is no statement in the rejection that Rens teaches producing a mammalian from the inseminated sperm. Secondly, the claims are drawn to methods of sorting sperm and not to methods of impregnating a mammal via insemination with sorted sperm. Thirdly, Rens (column 4) does teach that "A high speed sorter equipped with the nozzle of this invention increases the yield of sorted X- and Y-chromosome bearing sperm 10-fold and will make artificial insemination with sexed sperm a more feasible alternative to in vitro fertilization and embryo transfer or surgical insemination." If it is Applicants belief that the method of Rens does not result in the production of an

artificial insemination sample that is capable of fertilizing at least one egg then Applicants should have provided evidence to contradict this teaching of Rens. Further, the claims should have been amended to clarify that the invention was directed to a method of producing an equine via artificial insemination using sex-sorted sperm. However, Applicants have not provided any evidence to show that sperm sorted using the method of Rens could not be used to fertilize at least one egg and the claims are not limited to methods of producing an equine or methods of fertilizing an egg, but rather are drawn to methods of sorting equine sperm cells.

THE FOLLOWING ARE NEW GROUNDS OF REJECTION NECESSITATED BY APPLICANTS AMENDMENTS TO THE CLAIMS:

6. Claims 136 and 137 are rejected under 35 U.S.C. 103(a) as being unpatentable over Rens in view of Wilhelm and further in view of Schmid (cited in the IDS).

The teachings of Rens and Wilhelm are presented above. The combined references do not teach the quantity of sex-sorted sperm that is present in the insemination sample.

Schmid teaches sorting equine sperm on the basis of X- and Y-bearing chromosomes. Schmid further teaches insemination of equine using 150,000 sex-sorted sperm.

In view of the teachings of Schmid, it would have been obvious to one of ordinary skill in the art at the time the invention was made to have modified the method of Rens and Wilhelm so as to have created an insemination sample containing 150,000 sorted sperm for the advantage that this would have provided a sample that would be effective

for inseminating equine. With respect to claim 137, Schmid teaches using a .050 ml sample for insemination and does not teach a .2 ml sample of sperm. However, it would have been obvious to one of ordinary skill in the art and well within the skill of the art to have selected an optimum volume of sperm in order to have provided an effective sperm sample to be used for insemination.

7. Claims 45-51, 136 and 137 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 45-51, 136 and 137 are indefinite. The claims are drawn to methods for sorting equine sperm. However, the final process step is one of establishing an equine artificial insemination sample. Methods of sorting sperm are not the same as methods of establishing artificial insemination samples containing sperm which are capable of fertilizing at least one egg within an equine. Accordingly, it is not clear as to where the claims are intended to be limited to methods of sorting equine sperm or to methods of establishing an equine artificial insemination sample containing sperm which are capable of fertilizing at least one egg.

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within